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MONOCLONAL ANTIBODY-MEDIATED CLEAN-UP PROCEDURE FOR THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF CHLORAMPHENICOL IN MILK AND EGGS

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SUMMARY

A simple, rapid and specific sample preparation method based on antibody-mediated clean-up for the determination of chloramphenicol (CAP) in milk and eggs was developed. Skimmed milk and centrifuged egg homogenates were filtered and directly applied to immunoaffinity columns which were prepared by coupling monoclonal antibodies against CAP to a carbonyldiimidazole-activated support. Using a 0.2 *M* glycine, 0.5 *M* NaCl (pH 2.8) solution as an eluent, the immunoaffinity columns can be used more than 30 times without a decrease in column capacity. In subsequent high-performance liquid chromatographic analysis, no matrix interferences were observed. Good recoveries were obtained at spiking levels of 1–100 μg kg^{-1}. Due to the high specificity of the clean-up procedure, the limit of detection can be lowered by increasing the test portion. Concerning milk, the limit of detection was successfully lowered to 20 ng kg^{-1} by increasing the test portion to 1 l (recovery 99%). The method was applied to eggs produced by hens treated with CAP. The results are compared with those obtained by solid-phase extraction using silica gel.

INTRODUCTION

Various immunological methods for the detection and determination of residues of the broad-spectrum antibiotic chloramphenicol (CAP) have been described[1–8]. In these methods, antibodies against CAP were used in the final analysis. Recently we have demonstrated that these antibodies can also be used for a very specific clean-up and concentration of this compound from aqueous extracts of swine muscle tissue before high-performance liquid chromatographic (HPLC) analysis[9].

Usually, immunoaffinity columns are prepared by coupling antibodies to a cyanogen bromide (CNBr)-activated support[10,11]. However, this procedure has some disadvantages[11]: charged isourea groups are formed which are responsible for undesirable ion-exchange effects, and the isourea linkage is rather unstable. Therefore attention was paid, by Bethell *et al.*[12] and also by Hearn *et al.*[13], to an alternative coupling procedure using carbonyldiimidazole (CDI). The urethane linkage formed

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by this coupling procedure proved to be much more stable (leak resistant) than the isourea linkage introduced by the cyanogen bromide coupling procedure. Moreover, the urethane linkage is uncharged[11,14]. Therefore, non-specific binding due to ion-exchange effects does not occur.

In our earlier procedure the immunoaffinity columns were prepared by coupling monoclonal antibodies against CAP to a CNBr-activated support[9]. No decrease in CAP recovery at the 10 μg kg^{-1} level was observed after using these columns eleven times. On closer investigation, however, the column capacity was found to be decreased. Nevertheless, this capacity was high enough to guarantee good recoveries at the 10 μg kg^{-1} level. When the immunoaffinity columns were reused more often a continuous decrease in the column capacity led to lower recoveries. For that reason a CDI-activated support, which is now commercially available, was chosen for the preparation of the immunoaffinity columns. Other aspects with respect to reuse of the columns were also studied, such as the type of eluent and storage of the columns. Moreover, factors influencing the binding of CAP to the immunoaffinity columns such as the bed volume and the CAP concentration on the CAP capture efficiency were also investigated.

This paper describes the modified antibody-mediated clean-up procedure for the determination of CAP in eggs and milk. The method was also applied to eggs of hens to which CAP had been administered. The results obtained were compared with those obtained by a solid-phase extraction (SPE) procedure developed earlier[15].

EXPERIMENTAL

Reagents and chemicals

Water was purified by demineralization (conductivity <1 μS). Glycine and CAP were from Sigma (St. Louis, MO, U.S.A.), ammonium acetate, hexane, hydrogen chloride, sodium monohydrogenphosphate, potassium chloride, silica gel (average particle diameter 40 μm, for flash chromatography) and sodium acetate from Baker (Phillipsburgh, NJ, U.S.A.), ammonium sulphate, boric acid, citric acid monohydrate, isooctane, sodium chloride, sodium hydrogencarbonate, potassium dihydrogenphosphate from Merck (Darmstadt, F.R.G.), acetonitrile and methanol (both HPLC grade) from Rathburn (Walkerburn, U.K.), sodium azide from BDH (Poole, U.K.) and carbonyldiimidazole (CDI)-activated trisacryl GF-2000 (in acetone slurry) and bicinchoninic acid (BCA) protein assay reagent from Pierce (Rockford, IL, U.S.A.). Filter-paper circles (S&S 589.1, diameter 90 mm; S&S 589.3, diameter 125 mm and S&S 589.1/2, diameter 125 mm) were obtained from Schleicher and Schüll (Dassel, F.R.G.). The 125-ml polypropylene beakers were from Sarstedt (Eindhoven, The Netherlands).

A standard solution was prepared by dissolving 25.0 mg of CAP in 10.0 ml of methanol. Working standards for HPLC were prepared in the range of 10–1500 ng ml^{-1} by diluting the standard solution in the HPLC eluent. Spiking solutions containing 0.10, 1.00 and 10.00 μg ml^{-1} of CAP were prepared by diluting the standard solution in methanol. The mobile phase solvent for HPLC was acetonitrile–0.01 *M* sodium acetate buffer pH 5.4 (25:75, v/v).

Concentrated phosphate-buffered saline (PBS) was prepared by dissolving 80 g of NaCl, 14.33 g of $Na_2HPO_4 \cdot 2H_2O$, 2 g of KH_2PO_4, 2 g of KCl and 2 g of NaN_3 in

1 l of demineralized water. The PBS (pH 7.4; 0.1368 *M* NaCl, 0.0015 *M* KH_2PO_4, 0.0081 *M* $Na_2HPO_4 \cdot 2H_2O$, 0.0027 *M* KCl, 0.0031 *M* NaN_3) was prepared by diluting concentrated PBS 1:10 in demineralized water.

The coupling buffer solution (pH 8.5) was 0.13 *M* boric acid. The blocking buffer solution (pH 8.0) contained 0.2 *M* glycine; the acetate buffer (pH 4.0) contained 0.1 *M* sodium acetate and 0.5 *M* sodium chloride. Monoclonal antibodies against CAP are described below. The eluent used in the antibody-mediated clean-up procedure was a 0.2 *M* glycine, 0.5 *M* sodium chloride (pH 2.8) solution.

Apparatus

The instruments used were a Moulinette homogenizer (Moulinette, Gouda, The Netherlands), a spectrophotometer (Uvichem MK2; Rank Hilger, London, U.K.), a Prepspin 50 ultracentrifuge (Measuring and Scientific Equipment, Crawley, U.K.), a table centrifuge (Rotina/S; Hettich, Tuttlingen, F.R.G.), an Ultra-Turrax (Janke and Kunkel, Staufen, F.R.G.), a shaking apparatus (Janke and Kunkel, Type S50), a Reacti-Vap evaporating unit Model 18780, connected to a Reacti-Therm heating module, Model 18790 (Pierce), a vortex mixer (Scientific Industries, Bohemia, NY, U.S.A.), a magnetic stirrer (Pt 800; Protherm, Etten-Leur, The Netherlands), a sintered-glass funnel, diameter 40 mm, porosity 16–40 μm (P. M. Tamson, Zoetermeer, The Netherlands) and Visking dialysis tubing size 2 18/32 in. (Medicell, London, U.K.).

In order simultaneously to perform the antibody-mediated extractions, a proportioning pump III from a Technicon AutoAnalyzer II system was used in combination with Tygon calibrated flow-rated pump tubes, flow-rate 1.20 ml min^{-1}, 0.056 in. I.D. (Technicon, New York, NY, U.S.A.). The pump tubes were connected to immunosorbent packed Econo-columns (No. 737-122, 10 cm × 0.7 cm; Bio-Rad Labs., Richmond, CA, U.S.A.). The HPLC system used was the same as that described earlier[15].

Preparation and purification of monoclonal antibodies

The preparation and production of the monoclonal antibodies against CAP were performed in the hybridoma laboratory of the Department of Infectious Diseases and Immunology (Faculty of Veterinary Medicine, University of Utrecht, The Netherlands). These monoclonal antibodies possess the immunoglobulin G_1 (IgG_1) isotype. They were originally selected for an enzyme-linked immunosorbent assay (ELISA) and purified by ammonium sulphate precipitation as described earlier[8], but with the aid of boric acid solution as a coupling buffer. After dialysis the concentration of monoclonal antibodies in the purified solution, expressed as the amount of IgG per millilitre, was determined spectrophotometrically using the BCA protein determination[16].

Preparation of immunoaffinity columns

The excess of acetone of the CDI-activated support was removed by suction on a sintered glass funnel. The gel was washed with five bed volumes of ice-cold water. A 10-ml volume of the drained gel cake was transferred to 20 ml of the purified monoclonal antibody solution containing 5 mg ml^{-1} IgG. In this aqueous medium the 10-ml gel cake was swollen to a volume of 15 ml. The gel suspension was gently mixed

in a 50-ml polypropylene tube for 24 h at 4°C using a shaking apparatus. The suspension was centrifuged at 300 g for 3 min; the supernatant was used for the determination of the coupling efficiency (see below). The pellet was gently mixed with 20 ml of blocking buffer for 2 h at room temperature using a shaking apparatus. The gel suspension was centrifuged at 300 g for 3 min. The pellet was successively washed with 20 ml of coupling buffer, 20 ml of acetate buffer and 20 ml of coupling buffer. Finally the gel was washed with 50 ml of PBS and transferred to the columns (bed volume 0.5 ml per column).

The efficiency of the monoclonal antibody conjugation to CDI-activated tris-acryl GF-2000 was determined from the concentration of IgG in the diluted purified monoclonal antibody solution before and after the coupling procedure. The concentration of IgG was determined as described above. The dynamic and specific column capacities were determined in an analogous manner to that described earlier[9]. Instead of elution with methanol, the saturated immunosorbent was eluted with 0.2 M glycine, 0.5 M NaCl (pH 2.8) as described.

Samples

Spiking studies. Full-cream milk and whole eggs were used for spiking studies. The milk and homogenized egg samples were spiked with CAP at 1, 10 and 100 μg kg^{-1} at least 15 min before sample preparation.

Animal studies. Four laying hens (Hubbard golden comet, 25 weeks old) were treated with CAP through their drinking water during 5 successive days. The dosages were 0.05 (group A) and 0.5 g l^{-1} (group B) respectively; two animals for each group. Two laying hens served as the control group. The eggs were collected each day. Eggs produced within one group on the same day were pooled and homogenized using an Ultra Turrax for 45 s. Portions were frozen until analysis.

Sample preparation (antibody mediated clean-up)

Milk. Approximately 10 g of homogenized milk were accurately weighed in a 20-ml polypropylene tube. The milk sample was centrifuged at 3000 g for 15 min. Fat (upper layer) was removed. The skimmed milk sample was filtered through S&S 595.½ filter-paper. The polypropylene tube was rinsed with 5 ml of PBS. The wash liquid was filtered through the same filter. The total filtrate was subjected to antibody-mediated clean-up.

Eggs. Approximately 10 g of spiked homogenized whole egg were accurately weighed in a 14-ml polycarbonate tube. With respect to samples in the animal study, however, smaller test portions must be used in some cases to avoid overloading of the immunoaffinity columns. For the egg homogenates from day 1 up to day 12 (group A), day 13 and day 14 (group A) and day 1 up to day 9 (group B) the amounts were lower (0.5, 1.0 and 5.0 g respectively). To these samples, blank egg homogenate was added up to 10 g.

The homogenates (spiking study and animal study) were centrifuged at 10 000 g for 10 min. The supernatant was filtered through S&S 589.1 filter-paper. The pellet was washed with 5 ml of PBS. The wash liquid was filtered through the same filter. The total filtrate was subjected to antibody-mediated clean-up.

Antibody-mediated clean-up. The total sample solution was pumped through the immunoaffinity column at a rate of 1.2 ml min^{-1} using a Technicon proportioning

pump. The column was washed with 25 ml of PBS; CAP was eluted subsequently with 20 ml of glycine–NaCl eluent at a flow-rate of 1.2 ml min^{-1}. The eluate was collected in a 50-ml polypropylene tube. It was extracted twice with ethyl acetate (1 × 15 ml, 1 × 10 ml) using a shaking apparatus for 15 min. The upper organic layers were successively evaporated to dryness in a stream of nitrogen at 45°C using the evaporating unit and heating module (position 3, high). The residue was dissolved in 1 ml of the mobile phase solvent using a vortex mixer for 15 s. This solution was used for HPLC analysis.

Before regeneration, the risk of cross-contamination was minimized by washing the column with 20 ml of the glycine–NaCl eluent at a flow-rate of 1.2 ml min^{-1}. The columns were regenerated by washing with 20 ml of PBS at a flow-rate of 1.2 ml min^{-1}. If not in use the columns should be stored in PBS at 4°C. The columns were not allowed to run dry during the antibody-mediated clean-up and regeneration. If air-bubbles are present the immunosorbent should be shaken until a gel suspension is formed. Then the gel was allowed to settle again.

Sample preparation (solid-phase extraction)

Approximately 10 g of the homogenized egg sample were subjected to a solid-phase extraction procedure which comprises sonication-aided extraction with ethyl acetate, addition of hexane to the extract and cleaning up and concentration of the extract on a small column packed with silica gel. Compared to the solid-phase extraction described earlier for the determination of CAP in swine muscle tissue[15], some modifications were introduced[17]. After solid-phase extraction, all samples (dissolved in mobile phase solvent) were extracted three times with 1-ml volumes of isooctane. The remainder solution was subjected to HPLC analysis.

Chromatography

The samples were subjected to HPLC analysis. The HPLC conditions were as described earlier[15], except for the pH of the mobile phase. Aliquots of the sample and standard solution (40 μl) were injected by means of the loop injector. For low concentrations (below 10 μg kg^{-1}), 100-μl volumes were injected.

RESULTS AND DISCUSSION

Spiking studies

Recovery experiments were carried out on full-cream milk and eggs at spiking levels of 1, 10 and 100 μg kg^{-1}. The samples, including the blank samples, were subjected eight-fold to antibody-mediated clean-up followed by HPLC analysis according to the procedure described. The results are presented in Table I. At the spiking level of 1 μg kg^{-1} the recoveries are nearly 100%. However, at higher spiking levels the recoveries are somewhat lower. A logarithmic relationship exists between the spiking level and the recovery found, the coefficient of correlation being 1.0000 for milk and 0.9919 for egg.

The lowest standard deviations were obtained at the spiking level of 10 μg kg^{-1}. The higher standard deviations at 1 μg kg^{-1} may be attributed to the fact that the HPLC analysis was performed quite close to the limit of detection of the CAP standard, being 0.6 ng (signal corresponding three times the noise level). The cause of the

TABLE I

RECOVERY OF CHLORAMPHENICOL FROM SPIKED MILK AND EGGS

	Added ($\mu g\ kg^{-1}$)	*Recovery* (%)	*Standard deviation* (%)
Milk	1	100	8.5 (n = 8)
	10	91	5.5 (n = 8)
	100	82	7.4 (n = 8)
Eggs	1	98	12.2 (n = 7)[a]
	10	91	3.2 (n = 7)[a]
	100	80	6.1 (n = 8)

[a] One sample was lost during sample preparation.

higher standard deviations at the spiking level of 100 μg kg^{-1} and the lower recoveries at higher spiking levels will be discussed below. Typical chromatograms from spiked egg and milk samples are shown in Fig. 1. Very clean chromatograms were obtained. In this way, CAP can be determined in full-cream milk and eggs at the spiking level of 1 μg kg^{-1}.

Due to the absence of matrix interferences, a greater amount of sample can be subjected to antibody-mediated clean-up. This opens up the possibility to determine lower CAP contents. For example, a milk solution spiked with 20 ng kg^{-1} can be analysed when 1 l instead of 10 ml is subjected to antibody-mediated clean-up (recovery 99%). The chromatograms obtained after HPLC analysis were as clean as those in Fig. 1. In the analysis of egg homogenates, however, it was not possible to enlarge the test portion for antibody-mediated clean-up due to clogging of the column.

Antibody-mediated clean-up

Conjugation efficiency and column capacity. The efficiency of the monoclonal antibody conjugation to CDI-activated trisacryl GF-2000 was 59%. The protein loading was calculated as 3.10 mg of monoclonal antibody per ml of gel. The dynamic column capacity was found to be 2.73 μg of CAP per ml of gel, and the specific column capacity was 0.88 μg of CAP per mg of immobilized monoclonal antibody.

Reuse of immunoaffinity columns. To investigate the effect of reuse of immunoaffinity columns, the total column capacities were determined. Immunoaffinity columns with a bed volume of 1.5 ml were saturated by passing 20 ml of a 1 μg ml^{-1} CAP solution in PBS at a flow-rate of 1.2 ml min^{-1}. After washing with 25 ml of PBS, the columns were eluted with the glycine–NaCl solution or with methanol as described earlier[9]. The eluate was analysed for CAP by HPLC. After regeneration, the cycle (saturation, washing, elution, analysis and regeneration) was repeated several times. The experiments covered a period of 1 month. When not in use the columns were stored in PBS at 4°C.

The results are presented in Fig. 2. Glycine–NaCl does not significantly influence the column capacity during 33 cycles, whilst using methanol the column capacity decreased strongly. After 6 cycles only 15% of the original column capacity is left; after 17 cycles the column capacity is only 100 ng. Therefore glycine–NaCl solution is

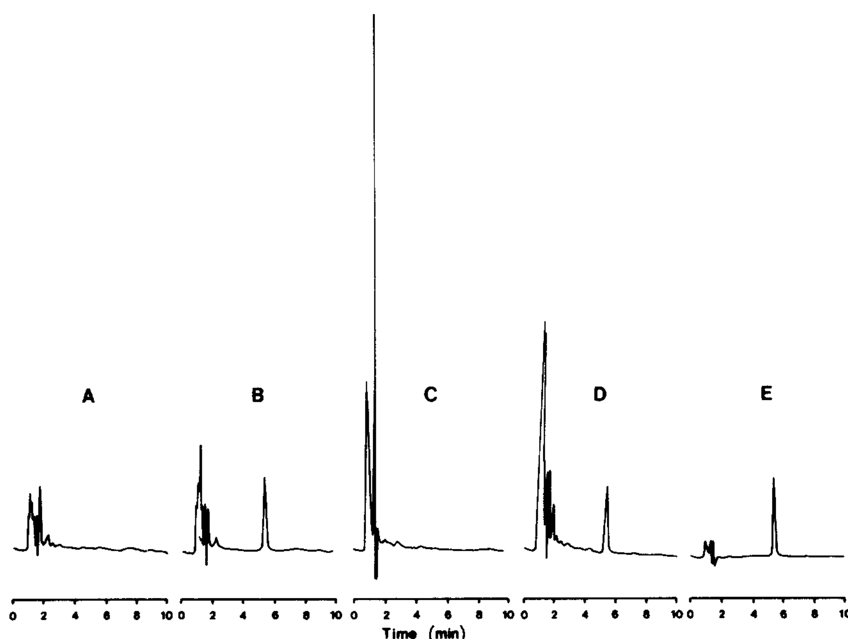

Fig. 1. Chromatograms of milk and egg samples purified by means of antibody-mediated clean-up followed by HPLC analysis. (A) Blank milk sample; (B) spiked (10 μg kg^{-1}) milk sample; (C) blank egg sample; (D) spiked (10 μg kg^{-1}) egg sample and (E) standard solution of CAP. Absorbance range settings: 0.016 a.u.f.s.

preferred as an eluent when the columns have to be reused many times. For single-use columns, methanol is the elution solvent of choice due to the easier pretreatment before HPLC analysis. Furthermore, the column capacity was slightly restored after storage in PBS (*viz.*, Fig. 2).

Capture efficiency. In the procedure described earlier for the determination of CAP residues in swine muscle tissue[9], addition of PBS to the aqueous tissue extracts before the antibody-mediated clean-up was necessary. In the case of milk and egg homogenates, however, the samples can be subjected to antibody-mediated clean-up directly after centrifugation and filtration. Extraction and dilution in PBS was not necessary for obtaining an high capture efficiency of CAP. However, as is seen from Table I, the recovery at the highest spiking level is lowered to 80%. This was caused by a lowering of the CAP capture by the column and not by incomplete elution. At that spiking level the amount of CAP for capture approximates the total column capacity for CAP.

To investigate how to improve the capture efficiency, in particular at amounts of CAP close to the column capacity, the following study was carried out. A fixed amount of CAP, *i.e.*, 1250 ng, was dissolved in respectively 2, 10 and 50 ml of PBS or skimmed milk. Antibody-mediated extractions were performed using columns with a 0.5-ml bed volume. For CAP in PBS the capture efficiency increased from 56% for the most concentrated solution (1250 ng CAP per 2 ml) to 90% for the most diluted solution (1250 ng CAP per 50 ml). For skimmed milk the same tendency was observed: 72% for the most concentrated solution and 88% for the most diluted solution.

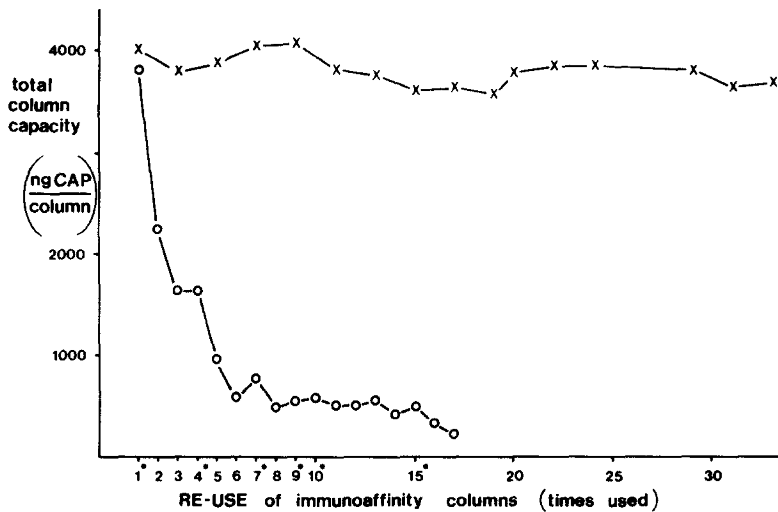

Fig. 2. The effect of storage of the immunosorbent in PBS at 4°C and of the type of eluent used on the total column capacity by reuse of the immunoaffinity columns. The successive antibody-mediated extraction cycles (saturation, washing, elution and regeneration) were performed with two identical immunoaffinity columns (bed volume 1.5 ml). One column was used for the methanol elution (○—○), the other column for the glycine–NaCl elution (× — ×). The numbers provided with an asterisk are the first capacity determinations performed on a new day. The capacity determinations were made over a period of 1 month.

Similar experiments were carried out using immunoaffinity columns with a bed volume of 1.0 ml.

An increase in the bed volume of the immunoaffinity columns from 0.5 to 1.0 ml slightly improved the capture efficiency at all concentrations. The results indicate that at a fixed bed volume a more efficient binding of CAP can be reached by lowering the CAP flux through the column or, in other words, when the same amount of CAP was extracted from a greater volume. This holds especially when the amount of CAP to be extracted approximates the total column capacity. This means that dilution of the sample solution which is subjected to antibody-mediated clean-up and/or lowering of the flow through the column will result in an higher recovery of CAP. However, these modifications were not introduced in the antibody-mediated clean-up described due to the increase in the analysis time. Apart from the lower recoveries at higher spiking levels, the higher standard deviation at the spiking level of 100 μg kg^{-1} can also be explained by the antibody-mediated clean-up described. If the amount of CAP which is subjected to antibody-mediated clean-up approximates the total column capacity, it is a matter of course that small changes in conditions (such as the bed volume, temperature and sample composition) cause greater differences in recovery.

By comparing the results of the capture of CAP from skimmed milk with those from PBS, it is remarkable that a more efficient capture is obtained from skimmed milk. In general, PBS is considered to give the optimum condition for antigen–antibody interaction.

TABLE II

CHLORAMPHENICOL CONTENT OF EGGS PRODUCED BY LAYING HENS TREATED WITH CHLORAMPHENICOL VIA THEIR DRINKING WATER DURING 5 SUCCESSIVE DAYS

AMC = Antibody-mediated clean-up; SPE = solid-phase extraction.

Days after starting the CAP treatment	*CAP dosage = 0.05 g l⁻¹*		*CAP dosage = 0.5 g l⁻¹*	
	AMC (*μg kg⁻¹*)	*SPE* (*μg kg⁻¹*)	*AMC* (*μg kg⁻¹*)	*SPE* (*μg kg⁻¹*)
1	41.8	62.0	568.1	835.6
2	48.8	58.9	775.5	884.0
3	47.5	71.3	861.2	1107.2
4	63.8	98.7	1214.8	1392.5
5	75.4	119.1	1321.9	1565.5
6	55.5	79.2	774.7	980.0
7	52.1	79.1	891.3	1021.2
8	37.3	61.4	854.4	997.2
9	27.2	57.5	1003.0	973.5
10	21.5	38.1	428.1	565.4
11	10.4	19.4	266.4	374.4
12	2.5	6.2	171.7	228.1
13	1.7	3.3	29.3	53.0
14	<1.0	<1.0	6.2	10.5
15	<1.0	<1.0	1.4	1.6
16	<1.0	<1.0	<1.0	<1.0
17	<1.0	<1.0	<1.0	<1.0

Animal study

The eggs of treated hens were analysed for CAP after antibody-mediated clean-up and solid-phase extraction using silica gel, respectively, according to the procedures described. The results shown in Table II indicate that, even after 1 day of CAP administration, considerable amounts of CAP were eliminated into eggs. CAP levels under 1 μg kg^{-1} were observed 14–16 days after starting the CAP treatment. All values were obtained by one replicate determination. The mean recovery for CAP-spiked eggs obtained after solid-phase extraction was identical to those described earlier[15] for spiked swine muscle tissue, *i.e.*, 79%. The CAP contents of eggs obtained by the solid-phase extraction procedure are corrected for this recovery. In the case of antibody-mediated clean-up, each value is corrected for a recovery obtained from the logarithmic relationship between the CAP content and recovery (see Spiking studies). The results obtained by the antibody-mediated clean-up and solid-phase extraction correlate well with each other (*viz.*, Fig. 3), the coefficient of correlation being 0.9932. It is remarkable, however, that nearly all results obtained with the aid of the antibody-mediated clean-up procedure are lower than those obtained by the solid-phase extraction procedure (*viz.*, Table II). The explanation for this phenomenon may be as follows: in the solid-phase extraction procedure the eggs are sonication-aided extracted with ethyl acetate. This solvent may cause a disruption of protein-bound drugs[18,19]. Therefore a more complete extraction of CAP may be possible. In the antibody-mediated extraction, on the other hand, the samples are applied to the

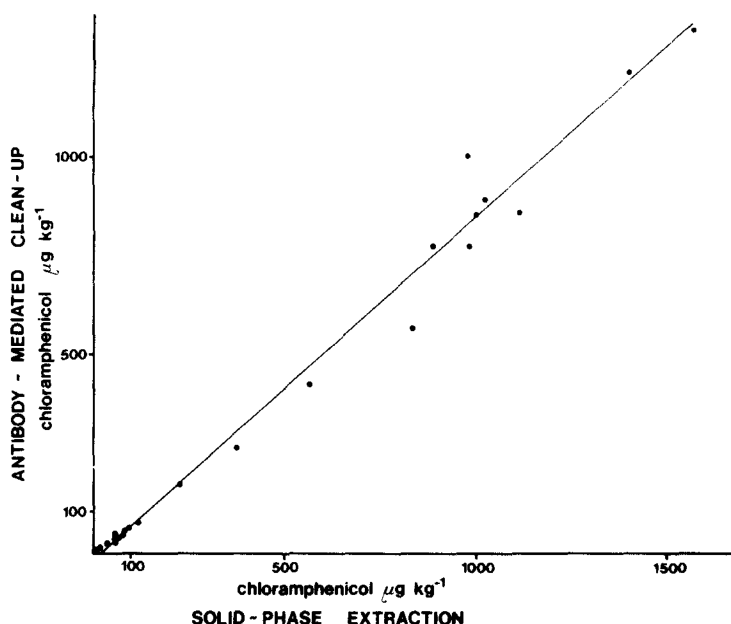

Fig. 3. Correlation between antibody-mediated cleaned and solid-phase extracted egg samples. Eggs were collected from treated hens; 0.05 and 0.5 g CAP l^{-1} drinking water during 5 successive days. The line is given by $y = 0.87x - 20.38$ ($n = 28$, correlation coefficient 0.9932).

immunoaffinity column after centrifugation and filtration. In that case no disruption of the protein-bound CAP may occur. It is probable that at least a part of the protein-bound CAP remains in the precipitate after centrifugation. Another possibility may be that protein-bound CAP cannot be bound by the immunoaffinity column. The cause of the lower results obtained with the aid of the antibody-mediated clean-up will be studied in detail.

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